IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application Number	:	09/982,531	Confirmation No.: 8179
Applicant	:	Astrid VRANG et al.	
Filed	:	October 19, 2001	
Title	:	IMPROVED FERMENTATION METHOD FOR PRODUCTION OF HETEROLOGOUS GENE PRODUCTS IN LACTIC ACID BACTERIA	
TC/Art Unit	:	1636	
Examiner:	:	Nancy Vogel	
Docket No.	:	54320.000011	
Customer No.	:	21967	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA. 22313-1450

DECLARATION UNDER 37 C.F.R. § 1.132

Sir,

I, Søren Michael Madsen, declare that:

1) I have received a Ph.D. degree in "Characterization of regulated promoters from Lactococcus" from The Technical University of Denmark, Lyngby, Denmark, 15 January 2001.

2) I am a Group Leader at, and I am employed by, Bioneer A/S, formerly named Bioteknologisk Institut. I have been associated with research in the field of molecular biology within the field of lactic acid bacteria for approximately 14 years.

3) My *curriculum vitae* providing details of experience is attached.

4) I am a named inventor of U.S. Patent Application Serial Number 09/982,531. Based on the academic training and professional experience, I consider myself a person of ordinary skill in the technology of lactic acid bacterial cells and modifications thereof, and I was such a person in 2000 when the Application was filed.

5) I am a named inventor on WO 98/10079 published on March 12, 1998 to Madsen *et al.*

6) I have read, and am familiar with, the following documents:

a) U.S. Patent Application No. 09/982,531 (hereinafter "the '531 application");

b) the Non-Final Office Action mailed October 12, 2005, in the '531 application (hereinafter the "Office Action");

c) WO 98/10079 published on March 12, 1998 to Madsen *et al.* (hereinafter "Madsen *et al.*");

d) Callewaert *et al.* (February 2000) "Bacteriocin Production with *Lactobacillus amylovorus* DCE 471 Is Improved and Stabilized by Fed-Batch Fermentation." Applied and Environmental Microbiology 66(2): 606-613 (hereinafter "Callewaert *et al.*");

e) Jensen *et al.* (December 1993) "Minimal Requirements for Exponential Growth of *Lactococcus lactis*." Applied and Environmental Microbiology 59(12): 4363-4366 (hereinafter "Jensen *et al.*");

f) de Vos (1999) "Gene expression systems for lactic acid bacteria." Current Opinion in Microbiology 2: 289-295 (hereinafter "de Vos");

g) van Asseldonk *et al.* (March 1993) "Cloning, Nucleotide Sequence, and Regulatory Analysis of the *Lactococcus lactis dnaJ* Gene." Journal of Bacteriology 175(6): 1637-1644 (hereinafter "van Asseldonk *et al.*"); and

h) Israelsen *et al.* (July 1995) "Cloning and Partial Characterization of Regulated Promoters from *Lactococcus lactis* Tn*917-lacZ* Integrants with the New Promoter Probe Vector, pAK80." Applied and Environmental Microbiology 61(7): 2540-2547 (hereinafter "Israelsen *et al.*").

7) It is my understanding that claims 1-11, 14, 17, 24, 27, and 30-45 of the '531 application were rejected in the Office Action under 35 U.S.C. § 103(a) as being unpatentable over Madsen *et al.* in combination with other references (Appendix A is a copy of these claims). Office Action at 2-11.

8) I understand that this is an "obviousness rejection" where it is alleged that the combination of Madsen *et al.* with the other references allegedly would have made "obvious" the subject

matter defined by a claim (or claims) in question to a person of ordinary skill in the technology in 2000. Office Action at 2-11.

9) I understand that in determining the differences between a reference and the claims under 35 U.S.C. § 103(a) the question is not whether the differences themselves would have been obvious but whether the invention defined in the rejected claim(s) as a whole would have been obvious. Manual of Patent Examining Procedure (M.P.E.P.) at § 2142.

10) I understand that three basic criteria must be met to support a *prima facie* case of obviousness: (a) there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine the reference(s) teachings; (b) there must be a reasonable expectation of success; and (c) the prior art reference (or references when combined) must teach or suggest all the claim features. M.P.E.P. at § 2143.

11) I have been asked to comment on whether Madsen *et al.* in various combinations with Callewaert *et al.*; Jensen *et al.*; de Vos; van Asseldonk *et al.*; and Israelsen *et al.* provides a suggestion or motivation, to modify Madsen *et al.*, presents a reasonable expectation of success, and teaches or suggests each and every limitation of the claims.

12) It was explained to me that claims 1 and 30 are independent claims and the remaining claims in the '531 application are dependent from the claims 1 and 30. It was further explained that if claims 1 and/or 30 would not have been obvious, then the dependent claims also would not have been obvious over the references relied upon in a particular rejection, because dependent claims include all features recited in the independent claims (in addition to those recited in the dependent claims).

13) I understand that this Declaration will be submitted in response to the Office Action.

14) Page 1 lines 14-17 and page 4 lines 4-11 of the '531 application teach that fermentation processes include batch, fed-batch, and continuous.

15) Conventional or batch cultivation is a process where all nutrients required during one run of cultivation, except for molecular oxygen in an aerobic process and chemicals for pH adjustments, are added to the medium before the cultivation is started. Therefore, batch cultivation is a "fixed-volume" system. Specification at 11 lines 5-8.

16) Fed-batch cultivation is a cultivation process where the volume increases as aliquots of fresh medium (such as glucose) are periodically added. Specification at Example 5.

17) Continuous cultivation is a cultivation process where all nutrients are continuously added to the cultivation container or bioreactor and fractions of the medium and/or cell culture are removed at the same flow rate as that of supplied nutrients to maintain a constant culture volume. Specification at 10 line 33 to 11 line 2; Example 8.

18) Previous methods of producing heterologous gene products using lactic acid bacteria as host cells are based on batch cultivation of the host cells in chemically undefined, nutrient rich media. Specification at 4 lines 3-6.

19) A number of considerations must be taken into account to select a suitable system for overexpression of a desired gene product. Specification at 1 lines 22-23.

20) Important issues include the yield of heterologous gene product required, costs of using the expression system, and the biological activity of the recombinant gene product produced in the production host. Specification at 1 lines 23-26.

21) Claims 1 and 30 of the '531 application state that the, "method of producing a heterologous peptide, polypeptide or protein in a lactic acid bacterium" occurs "under fed-batch or continuous cultivation conditions in a chemically defined medium".

22) As taught by the specification, a conventional batch process using a chemically defined, i.e., synthetic, medium, results in lower yields than using a conventional nutrient rich and chemical undefined medium. Inventors of the '531 application found that this could be overcome by performing the fermentation processing in a continuous process or fed-batch process. Specification at 4 lines 6-11.

23) The Office Action rejected claims 1, 3-10, 14, 17, 24, and 27 as obvious in view of the combination of Madsen *et al.* with Callewaert *et al.*; and Jensen *et al.* Claims 3-10, 14, 17, 24, and 27 are dependent from claim 1. I will focus my analysis on claim 1, the only independent claim. Office Action at 2-4.

24) The Office Action notes that, "[t]he difference between the reference [Madsen *et al.*] and the claims is that specific media components, including glucose, and controlled feeding of glucose in fed-batch or continuous culture, are recited." Office Action at 3.

25) Madsen *et al.* describes batch cultivation of recombinant lactic acid bacterial host cells for several uses, e.g., the manufacture of lactic acid bacterial starter cultures. See p. 7 and Example 11. The cells comprise a particular expression vector, including a particular promoter sequence element described by Madsen *et al.* See p. 6.

26) Madsen *et al.* does not discuss fed-batch or continuous cultivation systems.

27) Madsen *et al.* does not provide a suggestion or motivation to substitute the batch cultivation method taught by Madsen *et al.* with a fed-batch or continuous cultivation process to optimize expression of a desired gene product, as recited in claim 1 of the '531 application.

28) Since all three fermentation systems are distinct, it is my opinion that, absent an explicit teaching or a suggestion in Madsen *et al.*, it would not have been obvious to me, a person of ordinary skill in the art in 2000, to modify the bacterial cultivation system as taught by Madsen *et al.* to be fed-batch or continuous because Madsen *et al.* does not teach or suggest such modifications.

29) Madsen *et al.* in my opinion fails to provide a reasonable expectation of success in using a fed-batch or continuous system to cultivate a recombinant bacterium to produce a heterologous peptide, polypeptide, or protein because Madsen *et al.* does not disclose or suggest such systems.

30) The other references cited in the Office Action do not remedy these defects in Madsen *et al.*

31) Callewaert *et al.* discloses the use of fed-batch cultivation to produce amylovorin L471, an endogenous protein which is a hydrophobic bacteriocin, produced by *Lactobacillus amylovorus* DCE471, a strain of a naturally occurring bacterium. Id. at 606. Callewaert *et al.* also states that his results of fed-batch fermentation are "....interesting regarding the development of continuous fermentation processes." Id. at 612.

32) However, both Callewaert *et al.*'s teachings of fed-batch cultivation and comments on continuous cultivation were "...to improve and stabilize the [particular] bacteriocin production." Id. at 606, 612.

33) Thus, Callewaert *et al.* is directed to a different technology than claim 1 of the '531 application at least because the reference discusses methods of optimizing the production of an endogenous protein and not the expression of a heterologous protein. Id. at Figures 1-4.

34) Therefore, Callewaert *et al.* does not provide motivation or suggestion to modify Madsen *et al.* in any manner. In particular, I find no suggestion or motivation in Callewaert *et al.* to use fed-batch cultivation of any organism, other than *Lactobacillus amylovorus* DCE471. Callewaert *et al.* and Madsen *et al.* also fail to provide any motivation or suggestion to combine the two disclosures.

35) Further, this is an unpredictable art where methods developed for cultivating of non-transformed bacteria and their proteins do not necessarily translate to cultivation of a transformed bacterial host cell producing heterologous peptide, polypeptide, or protein. Specification at 3 lines 22-24.

36) For instance, in the '531 application we teach that batch cultivation producing heterologous gene products using lactic acid bacterial host cells in chemically defined media resulted in significantly lower yields than batch cultivation using conventional undefined medium. We discovered that using fed-batch or continuous cultivation processes overcame this problem. Specification at 4 lines 4-11.

37) An example of the difficulty and unpredictability in this art is demonstrated by Jensen *et al.* who describes a significant amount of work that was necessary to optimize the growth of non-transformed *Lactococcus lactis* subsp. *lactis*. Id. at Table 1-4.

38) Jensen *et al.* describes batch cultivation of *Lactococcus lactis* subsp. *lactis*, a non-transformed bacteria, but does not teach or suggest fed-batch or continuous cultivation. See pp. 4363-64. Jensen *et al.* teaches that the addition of 19 amino acids to a growth medium increased the growth rate of the bacteria. Id. Abstract and p. 4364.

39) Therefore, Jensen *et al.* does not provide motivation or suggestion to modify Madsen *et al.* in any manner. In particular, I find no suggestion or motivation in Jensen *et al.* to use fed-batch or continuous cultivation of any organism. I also found no suggestion or motivation in Jensen *et al.* or Madsen *et al.* to combine these two disclosures.

40) Since in my opinion Callewaert *et al.* and Jensen *et al.* individually fail to suggest or provide a motive to modify Madsen *et al.* or to combine their disclosures with Madsen et al., I also conclude that Callewaert *et al.* and Jensen *et al.* together fail to provide a suggestion or motivation to modify Madsen *et al.*

41) Furthermore, any combination of Madsen *et al.* with Callewaert *et al.* and Jensen *et al.* would not have suggested to me the use of fed-batch or continuous cultivation of recombinant bacteria in the context of producing a heterologous peptide, polypeptide, or protein, as defined in claim 1 of the '531 application.

42) The Office Action rejected claims 1-10, 14, 17, 24, and 27 as obvious in view of the combination of Madsen *et al.* with Callewaert *et al.*; Jensen *et al.*; and de Vos. The Office Action noted that the difference between Madsen et al., Callewaert *et al.* and Jensen *et al.*, and the claims is the use of a constitutive promoter in the claims. It was also stated that de Vos discloses constitutive promoters for the expression of genes in lactic acid bacteria. Office Action at 4-5. Claims 2-10, 14, 17, 24, and 27 are dependent from claim 1. I will focus my analysis on claim 1, the only independent claim.

43) As discussed above, Madsen *et al.* describes batch cultivation of recombinant lactic acid bacterial host cells for several uses, such as the manufacture of lactic acid bacterial starter cultures. Madsen *et al.* does not discuss or suggest fed-batch or continuous cultivation systems, and fails to provide a suggestion or motivation to substitute a fed-batch or continuous cultivation process for the batch cultivation method taught by him.

44) As also discussed above, Callewaert *et al.* discloses the use of fed-batch cultivation to produce amylovorin L471, an endogenous protein produced by *Lactobacillus amylovorus* DCE471, and Jensen *et al.* discusses batch cultivation of non-transformed bacteria, *Lactococcus lactis* subsp. *lactis,* but does not teach or suggest fed-batch or continuous cultivation. Id. at 4363-64.

45) For the reasons discussed above, in my opinion, the combination of Madsen *et al.*, Callewaert *et al.* and Jensen *et al.* is not warranted by their disclosures. Any possible combination of these three publications would not have suggested to me the method of claim 1, at least because the combination would have failed to include fed-batch or continuous cultivation in the context of producing a heterologous peptide, polypeptide or protein, as defined in claim 1 of the '531 patent. See paragraph 38, above.

46) de Vos describes gene expression systems for lactic acid bacteria, such as constitutive and inducible, but does not teach or suggest fed-batch or continuous cultivation. See p. 289.

47) In my opinion, de Vos fails to provide motivation or suggestion to modify Madsen *et al.* (alone or with the other references discussed in this rejection) in any manner. In particular, I find no suggestion or motivation in de Vos to use fed-batch or continuous cultivation of any organism.

48) Any combination of Madsen *et al.* with Callewaert *et al.*; Jensen *et al.*; and de Vos would not have suggested to me the invention of claim 1, at least because the combination would have failed to include or suggest the use of fed-batch or continuous cultivation of recombinant bacteria in the context of producing a heterologous peptide, polypeptide, or protein, as defined in claim 1.

49) The Office Action rejected claims 1-11, 14, 17, 24, and 27 as obvious in view of the combination of Madsen *et al.* with Callewaert *et al.*; Jensen *et al.*; and van Asseldonk *et al.* The Office Action noted that Madsen *et al.* and Jensen *et al.* were cited for the reasons previously discussed in the Office Action, and the differences between these references and the "instant claim" is the use of a particular peptide, *usp45*. Office Action at 5-6. Claims 2-11, 14, 17, 24, and 27 are dependent from claim 1. As with the other rejections, I will focus my analysis on claim 1, the only independent claim.

50) For the reasons discussed above, in my opinion, the combination Madsen *et al.*, Callewaert *et al.* and Jensen *et al.* is not suggested by these publications. It is also my opinion that any combination of the three publications would have failed to include all the features of claim 1 of the '531 application, at least because the combination would not have included fed-batch or continuous cultivation conditions in the context of a method for making a heterologous peptide, polypeptide or protein as described in claim 1.

51) van Asseldonk *et al.* describes the cloning and characterization of the *dnaJ* gene of *Lactococcus lactis* bacteria, but he does not teach or suggest fed-batch or continuous cultivation. van Asseldonk describes batch and plate-based cultivation conditions. See p. 1637. He also describes a transcriptional fusion between the *dnaJ* expression signals and a *usp45-amyS* secretion cassette, which caused an increase in α-amylase activity after heat shock induction. Id. at Abstract.

52) In my opinion, van Asseldonk *et al.* does not provide a motivation or suggestion to modify Madsen *et al.* in any manner. In particular, I find no suggestion or motivation in van Asseldonk *et al.* to use fed-batch or continuous cultivation of any organism at least because van Asseldonk describes batch and plate-based cultivation.

53) A combination of Madsen *et al.* with Callewaert *et al.*; Jensen *et al.*; and van Asseldonk *et al.* would not have suggested to me the use of fed-batch or continuous cultivation of recombinant bacteria in the context of producing a heterologous peptide, polypeptide, or protein as defined in claim 1.

54) The Office Action rejected claims 30, 32-39, and 41-45 as obvious in view of the combination of Madsen *et al.* with Callewaert *et al.*; Jensen *et al.*; and Israelsen *et al.* In the Office Action it was stated that the difference between Madsen *et al.* and the claims is the recitation of specific media components, including glucose and yeast extract, and controlled feeding of glucose in fed-batch or continuous culture. Office Action at 6-9. Further, it was noted, in pertinent part, that Israelsen *et al.* discloses a method of making a heterelogous protein using recombinant lactic acid bacteria, wherein the culture medium is supplemented with yeast extract and glucose. Id. at 8. Claims 32-39 and 41-45 are dependent from claim 30. I will focus my analysis on claim 30, the only independent claim.

55) I discussed Madsen *et al.* above, and opined that he fails to render obvious claim 1 of the '531 application. I also concluded that Callewaert *et al.* and Jensen *et al.* fail to provide the motivation or suggestion to modify Madsen *et al.*, particularly to eliminate the batch process taught by Madsen *et al.* and instead use continuous or fed-batch cultivations. For the reasons discussed above, it is also my opinion that any combination of Madsen *et al.*, Callewaert *et al.* and Jensen *et al.* would have failed to suggest to me the method of claim 1, at least because the combination would not have suggested fed-batch or continuous fermentation conditions. Claim 30 of the '531 application is similar to claim 1. In one aspect, claim 30 differs from claim 1 by requiring that the chemically defined medium is supplemental with yeast extract. For the same reasons as stated above, in my opinion any possible combination of Madsen *et al.*, Callewaert *et al.* and Jensen *et al.* would have failed to suggest to me the method of claim 30, at least because the combination would have lacked fed-batch or continuous fermentation conditions.

56) Israelsen *et al.* describes the construction of *Lactococcus lactis* Tn*917*-LTV1 integrants expressing β-galactosidase and utilizes batch cultivation but does not teach fed-batch or continuous cultivation. See pp. 2540; 2542.

57) From Israelsen *et al.* I do not discern any motivation or suggestion to modify Madsen *et al.*, or to combine Madsen *et al.*, Callewaert *et al.* and Jensen *et al.* in any manner. In particular,

I find no suggestion or motivation in Israelsen *et al.* to use fed-batch or continuous cultivation of any organism.

58) Accordingly any combination of Madsen *et al.* with Callewaert *et al.*; Jensen *et al.*; and Israelsen *et al.* would not have suggested to me the use of fed-batch or continuous cultivation of recombinant bacteria in the context of producing a heterologous peptide, polypeptide, or protein, as defined in claim 30.

59) The Office Action rejected claims 30-39 and 41-45 as obvious in view of the combination of Madsen *et al.* with Jensen *et al.*; Callewaert *et al.*; Israelsen *et al.*; and de Vos. Madsen *et al.*, Callewaert *et al.*, Jensen *et al.* and Israelsen *et al.* were cited for the same reasons summarized above. It was stated that the difference between Madsen *et al.*, Callewaert *et al.*, Jensen *et al.* and Israelsen *et al.*, and the claims is the use of a constitutive promoter. The Office Action stated that de Vos discloses constitutive promoters used for expression of genes in lactic acid bacteria. Office Action at 9-10. Claims 31-39 and 41-45 are dependent from claim 30. I will focus my analysis on claim 30, the only independent claim.

60) For the reasons discussed above, Jensen *et al.*, Callewaert *et al.* and Israelsen *et al.* fail to suggest the modification of Madsen *et al.*, or the combination of Madsen et al. with the three references. It is my opinion, as also discussed above, that any combination of these four references would not have made obvious to me claim 30 of the '531 application.

61) de Vos describes gene expression systems for lactic acid bacteria such as constitutive and inducible, but does not teach or suggest fed-batch or continuous cultivation. See p. 289.

62) In my opinion, de Vos fails to provide motivation or suggestion to modify Madsen *et al.* (alone or with the other three references discussed in this rejection) in any manner. In particular, I find no suggestion or motivation in de Vos to use fed-batch or continuous cultivation of any organism.

63) Accordingly, any combination of Madsen *et al.* with Jensen *et al.*; Callewaert *et al.*; Israelsen *et al.*; and de Vos would not have suggested to me the method of claim 30, at least because such combination would have failed to suggest the use of fed-batch or continuous

cultivation of recombinant bacteria in the context of producing a heterologous peptide, polypeptide, or protein, as defined in claim 30.

64) The Office Action rejected claims 30-45 as obvious in view of the combination of Madsen *et al.* with Callewaert *et al.*; Jensen *et al.*; Israelsen *et al.*; and van Asseldonk *et al.* Madsen *et al.*, Callewaert *et al.*, Jensen *et al.* and Israelsen *et al.* were cited for the same reasons as in previous rejections. It was noted that the difference between these four references and the "instant claim" is the use of a particular signal peptide, *usp45*. Office Action, at 10-11. Claims 31-39 and 41-45 are dependent from claim 30. I will focus my analysis on claim 30, the only independent claim.

65) For the reasons discussed above, in my opinion Callewaert *et al.*; Jensen *et al.* and Israelsen *et al.* fail to suggest a modification of Madsen *et al.*, or a combination of Callewaert *et al.*; Jensen *et al.* and Israelsen *et al.* with Madsen *et al.* to arrive at claim 30 of the '531 application. It is also my opinion that any combination of the four references would not have made obvious to me claim 30, as I also pointed out above.

66) van Asseldonk *et al.* describes the cloning and characterization of the *dnaJ* gene of *Lactococcus lactis* bacteria, but does not teach or suggest fed-batch or continuous cultivation. van Asseldonk describes batch and plate-based cultivation conditions. Id. at 1637. He also describes a transcriptional fusion between the *dnaJ* expression signals and a *usp45-amyS* secretion cassette, which caused an increase in L-amylase activity after heat shock induction.

67) In my opinion, van Asseldonk *et al.* does not provide motivation or suggestion to modify Madsen *et al.* in any manner. In particular, I find no suggestion or motivation in van Asseldonk *et al.* to use fed-batch or continuous cultivation of any organism, at least because he describes batch and plate-based cultivation.

68) Even any combination of Madsen *et al.* with Callewaert *et al.*; Jensen *et al.*; Israelsen *et al.*; and van Asseldonk *et al.* would not have suggested to me the use of fed-batch or continuous

cultivation of recombinant bacteria in the context of producing a heterologous peptide, polypeptide, or protein as defined in claim 30.

69) In my opinion, neither Madsen *et al.* nor any of the other references discussed above suggest the desirability of modifying Madsen *et al.*, for reasons discussed. It is my opinion that any of the combinations of the references would have failed to suggest to me the use of fed-batch or continuous cultivation conditions in a chemically defined medium to cultivate recombinant bacterium to produce heterologous peptide, polypeptide, or protein as defined in claims 1 and 30 of the '531 application.

70) The undersigned acknowledges that willful false statements and the like are punishable by fine or imprisonment, or both (18 U.S.C. § 1001), and may jeopardize the validity of the application or any patent issuing thereon. The undersigned declares further that all statements made herein of his/her own knowledge are true and that all statements made on information and belief are believed to be true.

I declare under penalty of perjury that the foregoing is true and correct.

Executed on 21 JULY 2006 Declarant's Signature: Jan Madsen

Home Address:

C/O Andersen

Dronning Elisabeths Allè 9

2300 København S

Denmark

APPENDIX A

Claim 1 (Previously Presented) A method of producing a heterologous peptide, polypeptide or protein in a lactic acid bacterium, the method comprising the steps of

(i) constructing a recombinant lactic acid bacterium comprising a nucleotide sequence coding for the heterologous peptide, polypeptide or protein and operably linked thereto, appropriate regulatory nucleotide sequences to control the expression of the coding sequence,

(ii) cultivating said recombinant bacterium under fed-batch or continuous cultivation conditions in a chemically defined medium, to express the nucleotide sequence, and

(iii) harvesting the recombinant bacterium or the peptide, polypeptide or protein,

wherein the concentration of glucose is kept at a pre-selected concentration of at least about 0.5 g/L by controlled feeding of glucose.

Claim 2 (Previously Presented) A method according to claim 1 wherein the recombinant bacterium comprises a constitutive promoter operably linked to the coding sequence.

Claim 3 (Previously Presented) A method according to claim 1 wherein the recombinant bacterium comprises a regulatable promoter operably linked to the coding sequence.

Claim 4 (Previously Presented) A method according to claim 3 wherein the regulatable promoter is regulated by accumulation of a metabolite intracellularly or in the medium.

Claim 5 (Previously Presented) A method according to claim 3 wherein the regulatable promoter is derived from a lactic acid bacterium.

Claim 6 (Previously Presented) A method according to claim 5 wherein the regulatable promoter is the P170 promoter disclosed in WO 98/10079 or a derivative thereof.

Claim 7 (Previously Presented) A method according to claim 3 wherein the promoter is introduced into the lactic acid bacterium on an autonomously replicating replicon.

Claim 8 (Previously Presented) A method according to claim 3 wherein the promoter is a promoter not naturally associated with the nucleotide sequence coding for the heterologous peptide, polypeptide or protein.

Claim 9 (Original) A method according to claim 1 wherein the heterologous peptide, polypeptide or protein is selected from the group consisting of an enzyme and a pharmaceutically active compound.

Claim 10 (Original) A method according to claim 1 wherein the coding nucleotide sequence is operably linked to a nucleotide sequence coding for a signal peptide (SP).

Claim 11 (Original) A method according to claim 10 wherein the signal peptide is selected from the group consisting of the Usp45 signal peptide and the signal peptide having the sequence MKFNKKRVAIATFIALIFVSFFTISSQDAQAAERS (SEQ ID NO: 1).

Claims 12-13 (Cancelled)

Claim 14 (Previously Presented) A method according to claim 1 wherein the control of feeding of glucose to the medium is linked to pH control.

Claims 15-16 (Cancelled)

Claim 17 (Previously Presented) A method according to claim 1 wherein the yield of heterologous peptide, polypeptide or protein is at least 5 mg/L.

Claims 18-23 (Cancelled)

Claim 24 (Previously Presented) A method according to claim 1 wherein the chemically defined medium is the medium comprising:

Component	Concentration, mM or +/-
L-Alanine	3.4
L-Arginine	1.1
L-Asparagine	0.8
L-Cysteine	0.8
L-Glutamate	2.1
L-Glutamine	0.7

Glycine	2.7
L-Histidine	0.3
L-Isoleucine	0.8
L-Leucine	0.8
L-Lysine-HCl	1.4
L-Methionine	0.7
L-Phenylalanine	1.2
L-Proline	2.6
L-Serine	2.9
L-Threonine	1.7
L-Tryptophan	0.5
L-Tyrosine	0.3
L-Valine	0.9
K_2SO_4	0.28[a]
KH_2PO_4/K_2HPO_4	4/6
Na-acetate	15
$CaCl_2$	0.0005[a]
$MgCl_2$	0.52[a]
$FeSO_4$	0.01[a]
Vitamins[b]	+
Micronutrients[a,c]	+
Citric acid	0.1

[a] From Neidhardt et al. J. Bacteriol. 119:736-747;

[b] Vitamins: 0.4 μM biotin, 10 μM pyridoxal-HCl, 2.3 μM folic acid, 2.6 μM riboflavin, 8 μM niacinamide, 3 μM thiamine-HCl and 2 μM pantothenate;

[c] Micronutrients: 0.003 μM $(NH_4)_6(MO_7)_{24}$, 0.4 μM H_3BO_4, 0.03 μM $CoCl_2$, 0.01 μM $CuSO_4$, 0.08 μM $MnCl_2$ and 0.01 μM $ZnSO_4$, or

wherein the components of said chemically defined medium are present in three-fold or five-fold amounts of the enumerated concentrations, except the phosphates and sodium acetate, the respective amounts of which are kept at the enumerated concentrations.

Claims 25-26 (Cancelled)

Claim 27 (Previously Presented) A method according to claim 1 wherein the chemically defined medium is the medium comprising:

Component	Concentration, mM or +/-
L-Alanine	3.4
L-Arginine	1.1
L-Asparagine	0.8
L-Cysteine	0.8
L-Glutamate	2.1

L-Glutamine	0.7
Glycine	2.7
L-Histidine	0.3
L-Isoleucine	0.8
L-Leucine	0.8
L-Lysine-HCl	1.4
L-Methionine	0.7
L-Phenylalanine	1.2
L-Proline	2.6
L-Serine	2.9
L-Threonine	1.7
L-Tryptophan	0.5
L-Tyrosine	0.3
L-Valine	0.9
K_2SO_4	0.28[a]
KH_2PO_4/K_2HPO_4	4/6
Na-acetate	15
$CaCl_2$	0.0005[a]
$MgCl_2$	0.52[a]
$FeSO_4$	0.01[a]
Vitamins[b]	+
Micronutrients[a,c]	+
Citric acid	0.1

[a] From Neidhardt et al. J. Bacteriol. 119:736-747;

[b] Vitamins: 0.4 μM biotin, 10 μM pyridoxal-HCl, 2.3 μM folic acid, 2.6 μM riboflavin, 8 μM niacinamide, 3 μM thiamine-HCl and 2 μM pantothenate;

[c] Micronutrients: 0.003 μM $(NH_4)_6(MO_7)_{24}$, 0.4 μM H_3BO_4, 0.03 μM $CoCl_2$, 0.01 μM $CuSO_4$, 0.08 μM $MnCl_2$ and 0.01 μM $ZnSO_4$;

wherein glucose is additionally included in the chemically defined medium in an amount in the range of 1-100 g/L, or

wherein glucose is additionally included in the chemically defined medium in an amount in the range of 1-100 g/L and wherein the components of said chemically defined medium are present in three-fold or five-fold amounts of the enumerated concentrations, except the phosphates and sodium acetate, the respective amounts of which are kept at the enumerated concentrations.

Claims 28-29 (Cancelled)

Claim 30 (Previously Presented) A method of producing a heterologous peptide, polypeptide or protein in a lactic acid bacterium, the method comprising the steps of

(i) constructing a recombinant lactic acid bacterium comprising a nucleotide sequence coding for the heterologous peptide, polypeptide or protein and operably linked thereto, appropriate regulatory nucleotide sequences to control the expression of the coding sequence,

(ii) cultivating said recombinant bacterium under fed-batch or continuous cultivation conditions in a chemically defined medium supplemented with yeast extract, to express the nucleotide sequence, and

(iii) harvesting the recombinant bacterium or the peptide, polypeptide or protein,

wherein the concentration of glucose is kept at a pre-selected concentration of at least about 0.5 g/L by controlled feeding of glucose.

Claim 31 (Previously Presented) A method according to claim 30 wherein the recombinant bacterium comprises a constitutive promoter operably linked to the coding sequence.

Claim 32 (Previously Presented) A method according to claim 30 wherein the recombinant bacterium comprises a regulatable promoter operably linked to the coding sequence.

Claim 33 (Previously Presented) A method according to claim 32 wherein the regulatable promoter is regulated by accumulation of a metabolite intracellularly or in the medium.

Claim 34 (Previously Presented) A method according to claim 32 wherein the regulatable promoter is derived from a lactic acid bacterium.

Claim 35 (Previously Presented) A method according to claim 34 wherein the regulatable promoter is the P170 promoter disclosed in WO 98/10079 or a derivative thereof.

Claim 36 (Previously Presented) A method according to claim 32 wherein the promoter is introduced into the lactic acid bacterium on an autonomously replicating replicon.

L-Methionine	0.7
L-Phenylalanine	1.2
L-Proline	2.6
L-Serine	2.9
L-Threonine	1.7
L-Tryptophan	0.5
L-Tyrosine	0.3
L-Valine	0.9
K_2SO_4	0.28[a]
KH_2PO_4/K_2HPO_4	4/6
Na-acetate	15
$CaCl_2$	0.0005[a]
$MgCl_2$	0.52[a]
$FeSO_4$	0.01[a]
Vitamins[b]	+
Micronutrients[a,c]	+
Citric acid	0.1

[a] From Neidhardt et al. J. Bacteriol. 119:736-747;

[b] Vitamins: 0.4 μM biotin, 10 μM pyridoxal-HCl, 2.3 μM folic acid, 2.6 μM riboflavin, 8 μM niacinamide, 3 μM thiamine-HCl and 2 μM pantothenate;

[c] Micronutrients: 0.003 μM $(NH_4)_6(MO_7)_{24}$, 0.4 μM H_3BO_4, 0.03 μM $CoCl_2$, 0.01 μM $CuSO_4$, 0.08 μM $MnCl_2$ and 0.01 μM $ZnSO_4$;

wherein the components of said chemically defined medium are present in three-fold or five-fold amounts of the enumerated concentrations, except the phosphates and sodium acetate, the respective amounts of which are kept at the enumerated concentrations.

Claim 45 (Previously Presented) A method according to claim 30 wherein the chemically defined medium is the medium comprising:

Component	Concentration, mM or +/-
L-Alanine	3.4
L-Arginine	1.1
L-Asparagine	0.8
L-Cysteine	0.8
L-Glutamate	2.1
L-Glutamine	0.7
Glycine	2.7
L-Histidine	0.3
L-Isoleucine	0.8
L-Leucine	0.8

L-Lysine-HCl	1.4
L-Methionine	0.7
L-Phenylalanine	1.2
L-Proline	2.6
L-Serine	2.9
L-Threonine	1.7
L-Tryptophan	0.5
L-Tyrosine	0.3
L-Valine	0.9
K_2SO_4	0.28[a]
KH_2PO_4/K_2HPO_4	4/6
Na-acetate	15
$CaCl_2$	0.0005[a]
$MgCl_2$	0.52[a]
$FeSO_4$	0.01[a]
Vitamins[b]	÷
Micronutrients[a,c]	÷
Citric acid	0.1

[a] From Neidhardt et al. J. Bacteriol. 119:736-747;

[b] Vitamins: 0.4 μM biotin, 10 μM pyridoxal-HCl, 2.3 μM folic acid, 2.6 μM riboflavin, 8 μM niacinamide, 3 μM thiamine-HCl and 2 μM pantothenate;

[c] Micronutrients: 0.003 μM $(NH_4)_6(MO_7)_{24}$, 0.4 μM H_3BO_4, 0.03 μM $CoCl_2$, 0.01 μM $CuSO_4$, 0.08 μM $MnCl_2$ and 0.01 μM $ZnSO_4$;

wherein glucose is additionally included in the chemically defined medium in an amount in the range of 1-100 g/L, or

wherein glucose is additionally included in the chemically defined medium in an amount in the range of 1-100 g/L and wherein the components of said chemically defined medium are present in three-fold or five-fold amounts of the enumerated concentrations, except the phosphates and sodium acetate, the respective amounts of which are kept at the enumerated concentrations.

Claim 37 (Previously Presented) A method according to claim 32 wherein the promoter is a promoter not naturally associated with the nucleotide sequence coding for the heterologous peptide, polypeptide or protein.

Claim 38 (Previously Presented) A method according to claim 30 wherein the heterologous peptide, polypeptide or protein is selected from the group consisting of an enzyme and a pharmaceutically active compound.

Claim 39 (Previously Presented) A method according to claim 30 wherein the coding nucleotide sequence is operably linked to a nucleotide sequence coding for a signal peptide (SP).

Claim 40 (Previously Presented) A method according to claim 39 wherein the signal peptide is selected from the group consisting of the Usp45 signal peptide and the signal peptide having the sequence MKFNKKRVAIATFIALIFVSFFTISSQDAQAAERS (SEQ ID NO: 1).

Claim 41 (Previously Presented) A method according to claim 30 wherein the control of feeding of glucose to the medium is linked to pH control.

Claim 42 (Previously Presented) A method according to claim 30 wherein the amount of yeast extract is in the range of 0.1-10 g/L.

Claim 43 (Previously Presented) A method according to claim 30 wherein the yield of heterologous peptide, polypeptide or protein is at least 5 mg/L.

Claim 44 (Previously Presented) A method according to claim 30 wherein the chemically defined medium is the medium comprising:

Component	Concentration, mM or +/-
L-Alanine	3.4
L-Arginine	1.1
L-Asparagine	0.8
L-Cysteine	0.8
L-Glutamate	2.1
L-Glutamine	0.7
Glycine	2.7
L-Histidine	0.3
L-Isoleucine	0.8
L-Leucine	0.8
L-Lysine-HCl	1.4

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